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DETERMINATION OF THE STRUCTURE OF DEXTRAN BY ^{13}C-NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

FRED R. SEYMOUR*, ROGER D. KNAPP**, AND STEPHEN H. BISHOP*

*Marrs McLean Department of Biochemistry, Baylor College of Medicine**;
*Division of Atherosclerosis and Lipoprotein Research, Baylor College of Medicine and Methodist Hospital***, *Texas Medical Center, Houston, Texas 77030 (U. S. A.)*

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ABSTRACT

The ^{13}C-n.m.r. spectra have been recorded for a series of dextrans whose structures, in terms of degree and type of branching, had previously been determined by methylation analysis. The spectra established that all observable linkages in these dextrans are α-linked. Correlation of the spectra with methylation data indicated that the 75–85-p.p.m. spectral region is diagnostic for establishing the presence of α-D-(1→2)-, α-D-(1→3)-, or α-D-(1→4)-linkages. Each chemical shift has been found to be temperature-dependent ($\Delta\delta/\Delta T$) when referenced to either the deuterium lock or an external standard (tetramethylsilane). All carbohydrate $\Delta\delta/\Delta T$ values are positive, and range from 0.01 to 0.03 p.p.m./°C. These values are considerably larger than analogous $\Delta\delta/\Delta T$ values previously observed for smaller molecules. Larger than average $\Delta\delta/\Delta T$ values are associated with the non-anomeric, sugar-linking carbon atoms.

INTRODUCTION

We report the recording and analysis of the ^{13}C-n.m.r. spectra of α-D-glucans that represent three classes of extracellular, microbial polysaccharides and that furnish examples of the various types of α-D-glucopyranosidic linkage. Five unusual examples of the dextran class of D-glucans differ in the identity and proportion of non-(1→6)-linkages combined with their predominant (1→6)-linkages. Two pullulans, whose structure appears to be representative of all of the well defined members of this class, have (1→4)-linked residues combined with (1→6)-linked residues in lesser proportion. Cyclohexaamylose was chosen as an example of a wholly (1→6)-linked polysaccharide. The dextrans employed were the same as those characterized through methylation–structural analysis by combined g.l.c.–m.s. of the peracetylated aldononitriles[1]. Structural data are summarized in Table I. These dextrans are essentially homogeneous fractions separated from the corresponding, polydisperse, high molecular-weight, native dextran[2] by fractionation with ethanol[3].

TABLE I

MOLE PERCENTAGE OF METHYLATED D-GLUCOSE COMPONENTS IN HYDROLYZATES OF PERMETHYLATED DEXTRANS[a]

Dextran-producing strain (NRRL number)	*Fraction*	*Methyl ethers of* D-*glucose*						
		2,3,4,6-	*2,3,4-*	*2,3,6-*	*2,4,6-*	*2,3-*	*2,4-*	*3,4-*
B-1351	S	5.8	83.3				10.5	0.3
B-1399	L	12.8	74.5				5.9[b]	6.8
B-1254	L	22.1	55.0	3.4		19.5		
B-1299	S	39.1	26.0					34.9
B-1355	S	6.9	46.9		35.0		11.2	

[a]Data in this Table from ref. 1. [b]Twice this proportion was detected in a duplicate determination.

The designations for the specific D-glucans refer to the NRRL number for the bacterial strain (*e.g.*, B-1229) producing the native dextran from which the specific dextran fraction was obtained. The bacterial strains employed were *Leuconostoc mesenteroides*, B-1299, B-1355, B-1399; *Streptococcus viridans*, B-1351; and *Streptobacterium dextranicum*, B-1254. In like manner, the pullulans employed (produced from NRRL strains of *Aureobasidium pullulans*, Y-6220 and Y-6992) have been shown* by methylation–structural analysis to contain (1→4)- and (1→6)-linked D-glucopyranosyl residues in the 2:1 ratio traditionally ascribed to pullulans. Optical rotations, methylation studies, and these ^{13}C-n.m.r. studies show that the D-glucans produced by Y-6220 and Y-6992 are identical. These pullulans provide examples of D-glucans having α-D-(1→6)-linkages present as a minor component, as well as comparison with α-D-(1→4)-linked D-glucans. Cyclohexaamylose [a glucan containing only α-D-(1→4)-linkages] was examined because of (*a*) its well defined and relatively simple structure[4], and (*b*) the fact that it has previously been studied[5,6] by ^{13}C-n.m.r. spectroscopy. Both the pullulan and cyclohexaamylose data are included, to provide chemical-shift data for the dextrans, as well as comparison with ^{13}C-n.m.r. data given in previous papers[7,8].

The sample parameters most obviously controllable are concentration, pH, and temperature. All of the measurements were made at similar concentrations (w/v) in deuterium oxide. Previous work[9,10] had indicated that chemical shifts can be dependent on pH, but the pH of these solutions was considered to be close enough to neutral not to require adjustment. The effect of temperature on ^{13}C-n.m.r. spectra of carbohydrates appears not to have been studied, and this effect is large enough to merit consideration.

We have found that ^{13}C-n.m.r. peaks of carbohydrates are displaced ~0.017 p.p.m./°C, indicating that, over a 60° increase in temperature, a peak will shift somewhat more than 1 p.p.m. Modern ^{13}C-n.m.r. instruments are accurate to within ±0.02 p.p.m.; therefore, a temperature change of 60° will result in a resonance

*M. Slodki, F. Seymour, and R. Plattner, unpublished results.

displacement twenty times greater than the error of the spectrometer. ^{13}C-N.m.r. spectra of carbohydrates are often published without any record of the sample temperature, and, when it is reported, the various determinations are often made at different temperatures.

It has previously been demonstrated[11,12] for carbohydrate compounds that the C-2, -3, -4, and -5 chemical-shifts are normally found in the 70–75-p.p.m. region. The anomeric carbon atom displays a downfield chemical-shift (~90 p.p.m.), and C-6, an up-field chemical-shift (~60 p.p.m.). Glycoside bond-formation causes the chemical shifts of the two carbon atoms involved to be displaced downfield by ~10 p.p.m., an effect equivalent to that of methyl ether formation[5,9]. Little α or β (carbon position relative to substituent addition) displacement has been noted for glycoside bond-formation.

For convenience, the spectra will be considered in four groups: (*a*) the anomeric region of 85 to 105 p.p.m. (mainly at 97–103 p.p.m., as there is only an infinitesimal proportion of reducing sugar in any of the polymers); (*b*) the 70–75-p.p.m. region normally associated with C-2,3,4, and 5, (*c*) the 60–70-p.p.m. region associated with bonded and nonbonded C-6 atoms, and (*d*) the 75–85-p.p.m. region into which the signals of bonded C-2, -3, -4, and -5 atoms are displaced. The close spacing of well defined peaks in the 70–75-p.p.m. region makes this a critical area for interpretation of ^{13}C-n.m.r. spectra.

An additional consideration for ^{13}C-n.m.r. analysis is the significance of relative peak-size. In contrast to ^{1}H-n.m.r. spectra, the peak area for ^{13}C-n.m.r. spectra does not necessarily reflect the population of atoms present in a specific environment. However, for carbohydrates, it has been shown that peak height is, *in general*, proportional to the number of carbon species present[12]. A number of arguments as to carbohydrate structure found in the literature have been based on relative peak-heights. Our data show that the relative peak-height can vary with the temperature. Therefore, we have not placed much emphasis on moderate differences in peak height. In deviation from the fundamental, α-D-(1→6), linear, dextran structure, we have examined D-glucans for which methylation data indicate branching, and, therefore, anomalously linked sugar units, in ratios greater than 1:4 (branch to backbone). For mole-ratio differences of this magnitude, we felt justified in identifying "minor" peaks and correlating these peaks to carbon atoms at points of branching.

RESULTS AND DISCUSSION

The effects of change of temperature on ^{13}C-n.m.r. spectra can be summarized as follows. Firstly, with increase in temperature, the line widths narrow, yielding a "sharper" spectrum; this change can be observed in the high-temperature spectra insets of the 70–75-p.p.m. regions of Figs. 1 and 2. Secondly, the relative intensity of various peaks can change with temperature; for the spectrum of dextran B-1355 fraction S (see Fig. 2), the peak at ~71.5 (27°) changes from a shoulder to become the prominent peak in the 90° spectrum. An analogous, although less dramatic,

change is observed for the C-6 peak of cyclohexaamylose. However, the relative intensities of the majority of the peaks change little with increasing temperature. Finally, as previously indicated, the chemical shift of each peak migrates downfield upon temperature increase.

For the D-glucans we have studied, the ratio of change in chemical shift to change in temperature ($\Delta\delta/\Delta T$) is positive, and of approximately equal magnitude for each peak. This change would imply that the total spectrum moves relative to the reference peak, a phenomenon that could be accounted for by a change in bulk magnetic susceptibility. In the spectra of dextran B-1355 fraction S and cyclohexaamylose, $\Delta\delta/\Delta T$ is constant between 27 and 90°. The average $\Delta\delta/\Delta T$ value for the various D-glucans is 0.017 p.p.m./°C, which is a useful correction-factor for the general comparison of carbohydrate spectra at different temperatures.

The actual $\Delta\delta/\Delta T$ value of each peak for which temperature-dependence data are available is shown in Table II, with the corresponding temperatures in Table III. The ^{13}C-n.m.r. data are in agreement with the previously determined, methylation–structural analysis data (see Table I) obtained for these D-glucans. A graph of the slopes for dextran B-1355 fraction S is shown, with the chemical shifts for each peak in the 70–75-p.p.m. regions at 27, 60, and 90° (see Fig. 3). The size of the circles

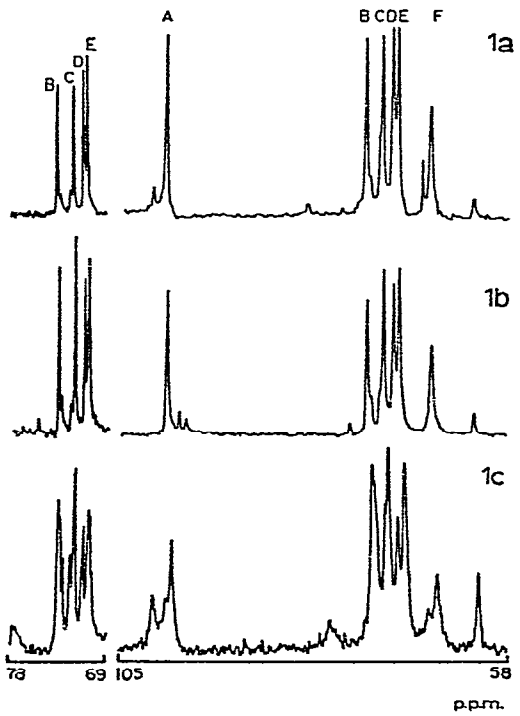

Fig. 1. ^{13}C-N.m.r. spectra at 27° with 69–78 p.p.m. (70°) spectra inset. [1a, Dextran B-1351 fraction S; 1b, dextran B-1399 fraction L; 1c, dextran B-1254 fraction L.]

TABLE II

CHEMICAL SHIFTS, RELATIVE INTENSITIES, AND $\Delta\delta/\Delta T$ FOR ^{13}C-N.M.R. SPECTRA OF D-GLUCANS

	Glucan *B-1351* *Fraction* *S* *α-Linkage* (*1→6*) & (*1→3*)	*B-1399* *L* (*1→6*) & (*1→2*)	*B-1254* *L* (*1→6*) & (*1→4*)	*B-1299* *S* (*1→6*) & (*1→2*)	*B-1355* *S* (*1→6*) & (*1→3*)	*Y-6992* (*1→6*) & (*1→4*)	*Cyclohexaamylose* (*1→4*)
							102.30 (1.21)
			101.03 (m, 1.11)			101.24 (1.05)	
	100.29 (m, 1.15)[a]				100.55 (1.09)	100.76 (1.36)	
			99.38 (m, 0.59)		99.02 (1.66)		
A	98.71 (M, 1.58)	98.71 (M, 1.44)	98.70 (M, 1.55)	98.74 (1.68)	98.93 (1.38)	98.95 (1.16)	
		97.22 (m, 1.77)		97.18 (2.00)			
		96.37 (m, 1.72)		96.40 (1.88)			
							82.13 (1.41)
	81.55 (m, 2.50)				81.60 (3.18)		
			79.54 (m, 2.01)			78.80 (3.07)	
						78.45 (2.41)	
		76.50 (m, 1.85)		76.42 (2.58)			
B	74.38 (M, 1.50)	74.40 (M, 1.42)	74.33 (M, 1.56)	73.84 (1.92)	74.36 (1.28)	74.38 (1.17)	74.24 (1.32)
							72.95 (1.43)
C	72.40 (M, 1.51)	72.40 (M, 1.42)	72.42 (M, 1.48)	72.39 (1.53)	72.62 (1.39)	72.51 (1.30)	72.62 (1.47)
D	71.16 (M, 1.52)	71.18 (M, 1.46)	71.26 (M, 1.46)	71.17 (1.68)	71.16 (1.39)	71.30 (1.63)	
E	70.52 (M, 2.01)	70.54 (M, 1.86)	70.44 (M, 2.10)	70.37 (2.10)	70.62 (1.37)	70.50 (1.75)	
	67.54 (m, 1.20)		67.64 (m, 1.01)			67.58 (2.37)	
F	66.55 (M, 2.29)	66.59 (M, 2.17)	66.55 (M, 2.30)	66.52 (2.42)	66.13 (2.73)		
	61.33 (m, 1.79)	61.38 (m, 1.87)	61.55 (m, 1.95)	61.35 (2.20)	61.35 (1.91)	61.65 (1.80)	
						61.45 (1.80)	
							61.36 (1.55)

[a]The first number is the chemical shift in p.p.m. relative to TMS. The letter stands for a major (M) or a minor (m) peak. The number in parentheses stands for the Δ p.p.m./ΔT (× 100) of the corresponding chemical-shift. The values of the chemical shifts listed are those observed at 27°.

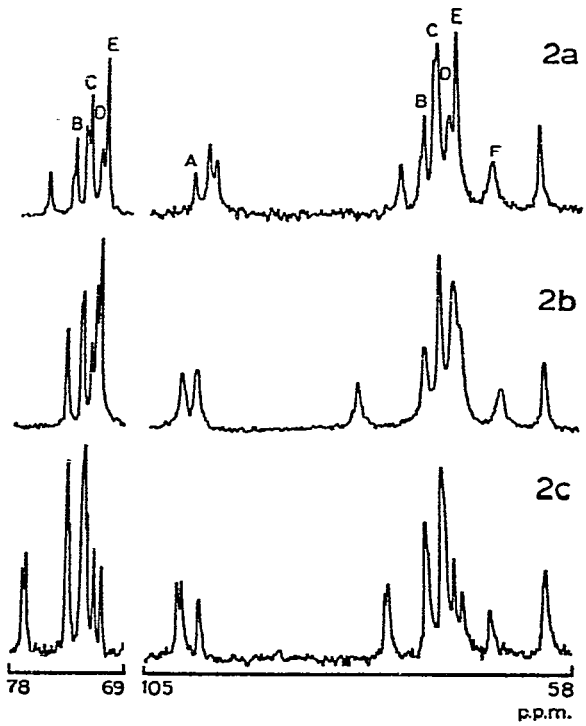

Fig. 2. ^{13}C-N.m.r. spectra at 27°. [2a, Dextran B-1299 fraction S (inset at 70°); 2b, dextran B-1355 fraction S (inset at 90°); 2c, pullulan Y-6992 (inset at 50°).]

TABLE III

TEMPERATURES USED FOR RECORDING THE SPECTRA OF D-GLUCANS

D-*Glucan*	*Spectrum temperature (°C)*				
B-1351 fraction S	27(57)[a]			70(84)	
B-1399 fraction L	27(50)			70(84)	
B-1254 fraction L	27(69)			70(11)	
B-1299 fraction S	27(4)			70(29)	
B-1355 fraction S	27(82)		60(50)		90(14)
Y-6220			50(25)		
Y-6992	27(18)		50(22)		
Cyclohexaamylose	27(1)	39(1)	50(1)	68(1)	85(1)

[a]The number in parentheses indicates acquisitions (in thousands) for that spectrum.

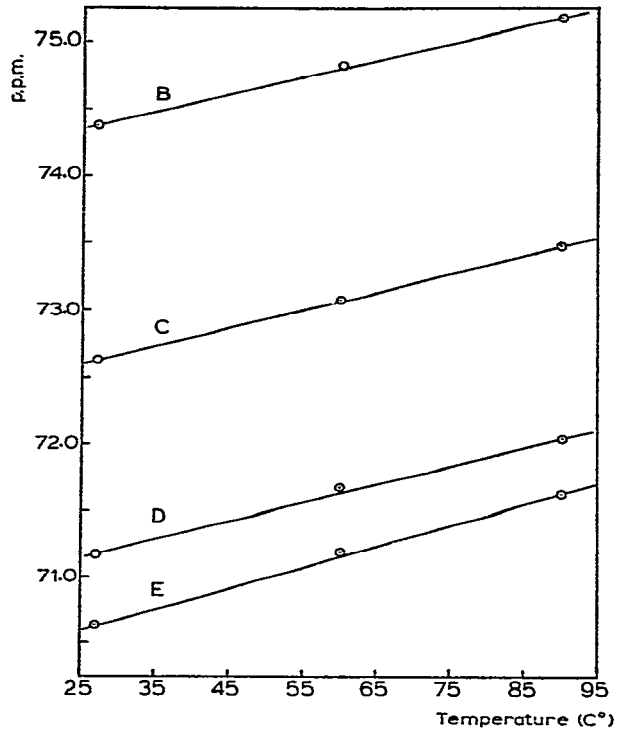

Fig. 3. Temperature dependence (°C) of the chemical shifts (p.p.m.) for the 70–75-p.p.m., ^{13}C-n.m.r. spectral region of dextran B-1355 fraction S.

indicates the error for temperature (±1°) and chemical shift (±0.02 p.p.m.); this shows that the change in $\Delta\delta/\Delta T$ is large compared to the experimental error, and that peak displacements are linear within the same error. Fig. 3 clearly shows the relationship of spectrometer accuracy, the close spacing of carbohydrate peaks, and the effect of $\Delta\delta/\Delta T$. At 90°, relative to ambient conditions, peak E has crossed over the low-temperature position of peak D, peak D approaches the low-temperature chemical-shift of peak C, and peak C approaches the low-temperature position of peak B. Despite this possibility for confusion, Fig. 3 contains peaks that have some of the smallest $\Delta\delta/\Delta T$ values observed for any carbohydrate peak. It should be noted (see Table II) that, with the exception of the anomeric carbon atoms, the largest $\Delta\delta/\Delta T$ values are associated with glycosidic linkage sites. Apparently, $\Delta\delta/\Delta T$ is a result of at least two effects, namely, bulk magnetic susceptibility and a contribution that is dependent (for each peak) on the structure of the compound.

Observation of n.m.r. chemical-shifts at different temperatures presents difficulties, because of the ability of the equipment to measure, precisely, the frequency difference between signals, but its inability to measure the absolute frequency of any given signal. This problem is normally solved by referencing the peaks to a known standard [*e.g.*, external, neat tetramethylsilane (TMS)]. When the XL-100-15

instrument is operated in the heteronuclear-lock mode, chemical shifts can be calculated by determining the offset of a reference signal for a given set of spectrometer conditions. In this case, internal deuterium oxide was the lock signal, and the offsets of internal 1,4-dioxane and external TMS (neat in a sealed, degassed, concentric, 5-mm tube) were measured from 29 to 80°. Chemical shifts could then be calculated from these offsets in the absence of a primary reference, the resonance of deuterium oxide being employed as a secondary reference. Therefore, if $\Delta\delta/\Delta T$ is observed for a carbon peak, three questions must be considered: first, is the D_2O peak, which is the actual reference, subject to $\Delta\delta/\Delta T$?; second, is a neat-TMS ^{13}C peak subject to $\Delta\delta/\Delta T$?; and finally, is the given ^{13}C-n.m.r. peak displaying $\Delta\delta/\Delta T$? For TMS, $\Delta\delta/\Delta T$ has been established to be a linear 0.012 p.p.m./°C relative to a constant, external frequency[13]. We have established that, relative to D_2O, $\Delta\delta/\Delta T$ for TMS ^{13}C is a linear -0.003 p.p.m./°C. For example, peak B of dextran B-1254 fraction L (see Table II) has a $\Delta\delta/\Delta T$ of 0.016 p.p.m./°C relative to the deuterium lock; this is a $\Delta\delta/\Delta T$ of 0.013 p.p.m./°C relative to an external-TMS signal, or a $\Delta\delta/\Delta T$ of 0.025 p.p.m./°C relative to a constant, external frequency. We have chosen to present our data in terms of actual chemical-shift from the deuterium lock-signal (in essence, considering the D_2O signal to be constant), but to express this value referenced to TMS by subtracting the accepted, TMS–1,4-dioxane frequency-difference at 27° from ^{13}C-n.m.r. spectra at all observed temperatures. This technique has the advantages of retaining the operational ease of the deuterium-lock reference, eliminating the necessity of introducing a $\Delta\delta/\Delta T$ correction-factor to the raw data, and presenting the data in readily recognizable quantities. Fortunately, the $\Delta\delta/\Delta T$ for deuterium oxide/TMS is small relative to other $\Delta\delta/\Delta T$ values; therefore, the actual values in Table II would differ little if either the D_2O signal or the TMS ^{13}C signal were considered to be constant.

Our general approach is to regard dextrans B-1399 fraction L and B-1351 fraction S as analogs of a linear, α-D-(1→6)-linked D-glucan, and to compare the spectra obtained for these dextrans to spectra of more highly branched D-glucans. For the less highly branched dextrans, there are six prominent chemical-shifts, which we designate as A through F. Peak A represents the anomeric carbon atom involved in α-D-(1→6)-linkages and, at 27°, appears at 98.7 p.p.m. Peaks B, C, D, and E are the four peaks that occur in the 70–75-p.p.m. region, and peak F represents the α-D-(1→6)-linked, C-6 chemical-shift at 61.4 p.p.m. (27°). For comparison of line width, selected regions of the spectra are shown at different temperatures (Figs. 1 and 2). The spectral temperatures for each polymer studied are summarized in Table III. All spectra were recorded at 27°, to provide the reference temperature for spectral comparison. Each D-glucan was measured at an additional temperature, and these data provided the $\Delta\delta/\Delta T$ values. In addition, spectra at intermediate temperatures were recorded for cyclohexaamylose and dextran B-1355 fraction S, which provided evidence for the linearity of the $\Delta\delta/\Delta T$ values; these data are summarized in Table I. Enhanced resolution allowed the identification of additional peaks at higher temperatures; these extra peaks are described in the individual polymer discussion (see later). The $\Delta\delta/\Delta T$

values are presented to three significant figures, so that the chemical shifts at higher temperatures can be accurately reconstructed, if so desired. The actual accuracy of the $\Delta\delta/\Delta T$ values is limited to the first two significant figures. We believe that we can distinguish between "major" peaks [the α-D-(1→6)-linked backbone] and "minor" peaks (branching) for the spectra of dextrans B-1254 fraction L, B-1351 fraction S, and B-1399 fraction L. Peaks A through F remain essentially constant with regard to chemical shift for all spectra except that of cyclohexaamylose.

The method of analysis for the minor peaks is as follows. The contributions from individual sugar units, in addition to those that belong to the fundamental, α-D-(1→6)-linked, poly-D-glucosyl backbone, can be considered as: (*a*) D-glucosyl residues involved in branching, (*b*) nonreducing D-glucosyl groups, and (*c*) non-α-D-(1→6)-linked, side-chain, D-glucosyl residues. For a first (and very workable) hypothesis, we assume a D-glucan to be a non-interacting assortment of alkyl (*i.e.*, methyl) ethers of methyl α-D-glucopyranosides. This hypothetical assortment may, for each dextran, be considered to be the reverse (methoxyl groups exchanged for hydroxyl groups) of the methylation monomers listed in Table I, in both type and molar proportion. For example, dextran B-1351 fraction S can be considered to be a mixture having a mole ratio of: 15 moles of methyl 6-*O*-methyl-α-D-glucopyranoside, one mole of methyl 3,6-di-*O*-methyl-α-D-glucopyranoside, and one mole of methyl α-D-glucopyranoside. Hence, minor peaks would be expected as contributions from the last two types. In a number of cases, these minor peaks would be hidden under prominent, major peaks. As the percentage of non-α-D-(1→6)-linkages increases, the minor peaks become equivalent to the major peaks. Ultimately, as in the case of pullulan Y-6992, the α-D-(1→6)-linked D-glucosyl residues provide minor peaks.

TABLE IV

CHEMICAL SHIFTS ASSIGNED TO SPECIFIC, CARBON POSITIONS

Values for methyl ethers of methyl α-D-glucopyranoside[a]			
Carbon position	*Unbound, p.p.m. (avg)*	*Bound, p.p.m.*	*Δ p.p.m.*
C-1	93	100.5	8.0
C-3	74	84.1	10.0
C-2	73	81.9	9.0
C-5	72		
C-4	71	80.5	10.0
C-6	62	72.6	11.0
Values[b] *from Table II*			
C-1 (A)		98.7	
C-3 (B)	74.3	81.6	7.3
C-2 (C)	72.2	76.5	4.3
C-5 (D)	71.2		
C-4 (E)	70.4	79.5	9.1
C-6 (F)	61.4	66.6	5.2

[a]Taken from ref. 5, Table III. [b]Based on α-(1→6)-dextrans at 25°.

For dextran B-1351 fraction S, there are the six major peaks (A through F) which we assume to be associated with the predominant, α-D-(1→6)-linked D-glucopyranosyl residues. These peaks occur, although with different relative intensity, in the spectra of all of the D-glucans reported in this paper, with the exception of that for cyclohexaamylose. Certain correlations can be made to previous work reported for methyl ethers of methyl α-D-glucopyranoside (see Table IV), assuming that our major peaks are caused by the specific carbon atoms of the methyl 6-*O*-methyl-α-D-glucopyranoside unit. However, certain conceptual difficulties arise. For example, it is strange that these peaks remain dominant even when a large degree of branching and many non-α-(1→6)-linkages occur.

With the exception of a branching detail for dextran B-1399 fraction L, all ^{13}C-n.m.r. data are in accord with previous methylation studies[1]. Although methylation data provide more-specific values for degree of branching, ^{13}C-n.m.r. spectroscopy provides certain information unobtainable by methylation analysis. ^{13}C-N.m.r. data provide very specific information about the anomeric linkages, as their configurations in D-glucans have previously been ascribed to chemical shifts of 97.5–101 p.p.m. (α) and 104–105 p.p.m. (β). Bulk properties, such as specific rotation, have indicated that the dextrans are primarily α-linked. Our data indicate that *all* glycosidic bonds, for branch points as well as for side-chain linkages, are α-linked. In addition, it should be noted that, in contrast to methylation analysis, ^{13}C-n.m.r. spectroscopy is nondestructive, provides data that are not dependent on hydrolysis techniques, and allows the identification of noncarbohydrate, organic material, if such be present.

The individual polysaccharides will now be considered in terms of their ^{13}C-n.m.r. spectra and their previously determined structures. Unless otherwise stated, the chemical shifts discussed will be those at 27°.

Dextran B-1351 fraction S

The ^{13}C-n.m.r. spectrum of the dextran produced by *Streptococcus viridans* provides well defined, A through F peaks. In addition, the 60–70-p.p.m. region shows two minor peaks: 61.33 p.p.m. is the nonbonded C-6 of the nonreducing, branch end-group, and 67.54 p.p.m. is C-6 of the 1,3,6-tri-*O*-substituted, branching, α-sugar residue. The minor anomeric peak corresponds to one of the minor sugar units, with the other minor anomeric peak hidden under peak A. The minor peak at 81.55 p.p.m. appears ~10 p.p.m. downfield of the 70–75-p.p.m. region, and represents C-3 of the 1,3,6-tri-*O*-substituted, branching α-sugar residue. The peak-width narrowing of the 70° spectrum allows the observation, but not the assignment, of a minor peak 0.36 p.p.m. upfield of peak B, and a minor peak 0.38 p.p.m. downfield of peak C.

Dextran B-1399 fraction L

The ^{13}C-n.m.r. spectrum of the dextran produced by *Leuconostoc mesenteroides* B-1399 is analogous to that of dextran B-1351 fraction S for the major peaks and the

terminal C-6 (61.37 p.p.m.). However, the remaining, minor peaks differ from those in the spectrum of dextran B-1351 fraction S. The 60–70-p.p.m. region shows no second, minor peak; this resonance is apparently hidden under peak F. The anomeric region contains two minor peaks, assigned to the 1,2,6-tri-*O*-substituted sugar residue and its appended, side-chain, D-glucosyl residue. The single resonance (76.50 p.p.m.) observed in the 75–85-p.p.m. region apparently arises from C-2 of the branching sugar. Previous methylation data[1] indicated that, in addition to (1→2)-branching, a somewhat lesser extent of (1→3)-branching occurs. In light of the radical differences between the minor peaks in the spectra of the dextran B-1351 fraction S and dextran B-1399 fraction L, and the absence of any peaks at 100.29, 81.55, and 67.54 p.p.m. in the spectrum of the latter, it is concluded that dextran B-1399 fraction L contains few, if any, 1,3,6-tri-*O*-substituted D-glucose residues. The 70° spectrum shows additional, minor peaks 0.35 p.p.m. upfield of peak B, and, in the 60–70-p.p.m. region, a peak 0.08 p.p.m. upfield of major peak F.

Dextran B-1254 fraction L

The dextran produced by *Leuconostoc mesenteroides* B-1254 presents a case of intermediate branching, represented by three α-D-(1→6)-linked D-glucosyl residues, a 1,4,6-tri-*O*-substituted sugar residue, and a nonreducing D-glucosyl group. The 27° spectrum again shows peaks A through F as dominant features. Although two additional, minor peaks appear in the 60–70-p.p.m. region (due to branching and nonreducing end-group sugars), C-6 of the nonreducing end-group gives as intense a peak as the C-6 peaks of the three α-D-(1→6)-linked D-glucosyl residues; this is not unexpected, as increased freedom of intermolecular motion of a substituent (*i.e.*, the sugars belonging to branch chains) can result in more-intense peaks[11]. Again, this finding further serves to illustrate the difficulties involved when peak intensities are invoked in structural determinations. The anomeric region also shows two minor peaks associated with the branching and nonreducing-terminal sugars. The peak displaced into the 75–85-p.p.m. region (79.54) is assumed to be the C-4 atom involved in branching. The 70° spectrum shows additional, minor peaks 0.47 p.p.m. upfield of peak B, 0.41 p.p.m. downfield of peak C, and 0.30 p.p.m. upfield of peak D.

Dextran B-1299 fraction S

Our ^{13}C-n.m.r. spectrum of the dextran produced by *Leuconostoc mesenteroides* B-1299 differs in certain details from that of a previous study[14]. Usui *et al.*[14] based ^{13}C-n.m.r. structural assignments on the premise that dextran B-1299 fraction S contains two backbone, α-D-(1→6)-linked D-glucosyl residues for each branching and nonreducing-terminal sugar. Our methylation data[1] indicated approximately equal amounts of nonreducing terminal, branching, and α-D-(1→6)-linked backbone sugars. Our ^{13}C-n.m.r. spectrum (see Fig. 2) is in agreement with the more highly branched structure. Although our ^{13}C-n.m.r. spectra differ somewhat from those of Usui *et al.*[14] in relative intensities of individual peaks in the anomeric region, this is probably attributable to our greater signal-to-noise ratio. We found only one peak in the

75–85-p.p.m. region, and only two in the 60–70-p.p.m. region. The 70° spectrum shows an additional peak, 0.30 p.p.m. downfield of peak C.

On comparing the spectrum of dextran B-1299 fraction S with that of dextran B-1399 fraction L, several similarities became apparent. The minor peaks of dextran B-1399 fraction L, attributed to 1,2,6-tri-*O*-substituted D-glucosyl residues and nonreducing terminal sugars, are all present, although greatly enhanced in the spectrum of dextran B-1299 fraction S. The C-6 chemical-shifts of both of the 1,2,6-tri-*O*-substituted D-glucosyl residues and the nonbranching, α-D-(1→6)-linked D-glucosyl residues are assumed to be represented by a single peak (66.59 p.p.m.), and the remaining, more-intense peak in this region (61.38 p.p.m.) is assigned to C-6 of the nonreducing end-group. This increased intensity for a terminal, side-chain, C-6 peak is in accord with the effect observed for dextran B-1254 fraction L. Comparison of the spectra of dextrans B-1399 fraction L and B-1299 fraction S also shows that the chemical shifts for the anomeric carbon atoms, for linked C-2, and for C-6 atoms agree within experimental error.

Dextran B-1355 fraction S

Methylation analysis indicates that the dextran produced by *Leuconostoc mesenteroides* B-1355 contains (1→6)- and (1→3)-linked D-glucosyl residues, with 1,3,6-tri-*O*-substituted D-glucosyl residues. In degree of branching and types of linkages, dextrans B-1351 fraction S and B-1355 fraction S have strong similarities. However, in contrast to dextran B-1351 fraction S, dextran B-1355 fraction S contains unbranched (1→3)-linkages, and a combination of methylation[1] and acetolysis data[15] indicated that (1→3)- and (1→6)-linkages often alternate. This dextran, then, contains four types of sugar unit: equal proportions of 1,3,6-tri-*O*-substituted D-glucosyl residues and nonreducing terminal sugars, with roughly equal and approximately four times the proportion of (1→3)- and (1→6)-linked D-glucosyl residues. For dextrans B-1351 fraction S and B-1355 fraction S, the correlation of chemical shifts for anomeric, C-3, and C-6 peaks is very good. The close correlation of the C-3 (81.60 p.p.m.) peak is interesting, for, in the spectrum of dextran B-1351 fraction S, this peak is contributed only by the 1,3,6-tri-*O*-substituted D-glucosyl residues, whereas, for dextran B-1355 fraction S, the major contribution comes from the (1→3)-linked D-glucosyl residues. The 70–75-p.p.m. region of dextran B-1355 fraction S contains extra peaks (apparent at high temperature), which indicate a contribution of the (1→3)-units. The 60–70-p.p.m. region offers several curious points. First, it is very simple, containing only two peaks (when it might have had four). Second, the 67.54-p.p.m., minor peak observed in the spectrum of dextran B-1351 fraction S is missing, although the percentage of branch units in the two D-glucans is about the same. Third, in like manner, methylation analysis indicates that the total number of nonreducing D-glucosyl groups is about the same for both dextrans, yet the intensity of the unbound C-6 peak (61.38) of the dextran B-1355 fraction S is much greater in the corresponding peak for the dextran B-1351 fraction S. These observations are not readily rationalized. The 90° spectrum of dextran B-1355 fraction S shows two

intense additional peaks, 0.18 p.p.m. downfield of peak C and 0.51 p.p.m. downfield of peak D. It is possible that the marked temperature-change for the 70–75-p.p.m. region, compared to that of the more linear dextrans, reflects the greater structural complexity of this polysaccharide.

Pullulans Y-6220 and Y-6992

As strains Y-6220 and Y-6992 of *Aureobasidium pullulans* produce typical pullulans which give essentially identical ^{13}C.-n.m.r. spectra, only one of the spectra is shown in Fig. 2. The spectra are also apparently identical to that published[7] for a glucan isolated from *Tremella mesenterica* NRRL Y-6158. The spectra were recorded at 50°, to provide direct correlation to the previous work, and they confirm that the pullulans are exclusively α-linked. The splitting (101 p.p.m.) of the anomeric carbon atom engaged in the (1→4)-linkage, which provided the basis for the elegant, structural explanation of regularly repeating (1→4)- and (1→6)-linked D-glucosyl residues, is also observed in our spectra. The linked, C-4 chemical-shift (79 p.p.m.) is also split, apparently for the same reasons[8]. This (1→4)-linkage is of interest for comparison with the spectra of dextran B-1254 fraction L and the completely α-D-(1→4)-linked cyclohexaamylose. Although the agreement between various peaks is not as good as for previous pairs, the peak in the 75–85-p.p.m. region, assumed to be caused by the linked C-4 atom, shows wide variation. For δ values in the 70–75-p.p.m. region, the difference observed between the spectra of dextran B-1254 fraction L and pullulan may lie in our comparing 1,4-di-*O*- to 1,4,6-tri-*O*-substituted residues. The divergence of the cyclohexaamylose data is discussed next.

Cyclohexaamylose

The ^{13}C-n.m.r. spectrum of this compound was measured at approximately 10° intervals from 27 to 85°, and the change in chemical shift *vs.* temperature ($\Delta\delta/\Delta$T) was found to be linear within the error of the spectrometer. In contrast to those of the other D-glucans, $\Delta\delta/\Delta$T was almost the same for all six peaks. The ^{13}C-n.m.r. spectra of this compound[5] and an analog[7] have previously been reported. On the basis of our temperature plots, it would appear that the spectrum of Usui *et al.*[5] was recorded at 75°, and that of Iwakura *et al.*[6] at 25°. The extremely narrow line-widths of the cyclohexaamylose spectra, when compared to those of the other ^{13}C-n.m.r. spectra, confirm that each D-glucose unit is in an extremely uniform chemical environment. At 27°, the relative intensities of all peaks were approximately the same, except for that at 61.36 p.p.m., which was approximately two-thirds the height of the others. Upon increase in temperature, the relative intensity of this weak peak steadily increased until, at 85°, the peak intensities for all six carbon atoms were approximately equal. In conjunction with the $\Delta\delta/\Delta$T studies for cyclohexaamylose, the chemical shift of a sealed, concentric, capillary tube of 1,4-dioxane was observed. The 1,4-dioxane shift was 67.43 p.p.m. (39°) with a linear $\Delta\delta/\Delta$T of 0.010.

It is possible that the similarity of all $\Delta\delta/\Delta$T values for cyclohexaamylose is another indication of the rigidity[4] of this annular system. The C-4 value (82.47 p.p.m.)

is curiously large when compared with those for pullulans Y-6992 and Y-6220 and dextran B-1254 fraction L, and does not exhibit the large $\Delta\delta/\Delta T$ value normally associated with these glycosidically linked carbon atoms. It is possible that an unusually large $\Delta\delta/\Delta T$ value is a reflection of increasing bond-strain, a condition which could occur in branched polymers, as increased thermal agitation at higher temperatures would strain the branching position. This concept is buttressed by the large, low-temperature, chemical-shift value for cyclohexaamylose, which may be evidence of the considerable strain on the (1→4)-linkages and the fact that the inherent rigidity of the annulus allows little difference in ring strain at higher temperatures.

A recent report[16] dealt with the $\Delta\delta/\Delta T$ values of some small, cyclic, aliphatic compounds, and found $\Delta\delta/\Delta T$ values (relative to TMS) of 0.001 to 0.006 p.p.m./°C, which would be equivalent to 0.004 to 0.009 p.p.m./°C relative to D_2O. These values are all significantly smaller than the $\Delta\delta/\Delta T$ value found for the carbohydrate peaks. The temperature-dependence of ^{13}C-n.m.r. shifts for pentylcyclohexane was of interest as the open-chain carbon atoms display negative $\Delta\delta/\Delta T$ values, whereas the ring-carbon atoms show the positive $\Delta\delta/\Delta T$ values observed for our pyranoside-ring polymers. On this basis, the nonlinked, exocyclic, C-6 atoms might be expected to have smaller than average $\Delta\delta/\Delta T$ values; however, they do not.

Certain generalizations can be drawn from these results. For an initial comparison, data for various positions of specifically methylated methyl α-D-glucopyranoside have been abstracted from the paper by Usui *et al.*[5] (their Table 3). In that paper, various chemical-shifts were assigned to the D-glucopyranoside carbon atoms on the basis of extensive mono-*O*-methyl substitution. We averaged the values found for non-bound carbon atoms of the various, substituted methyl α-D-glucopyranosides, and now list, in Table IV, the specific chemical-shifts for the methyl ethers of methyl α-D-glucopyranoside. The change in chemical shift upon methylation was also calculated for each carbon position (the data are presented in Table IV). Our D-glucan data are treated in an analogous manner. The major chemical-shifts are correlated in decreasing p.p.m. values, and assigned the same order of D-glucopyranoside carbon atoms as the monomers mentioned. The relative spacing of the monomer peaks and the polymer A through F peaks is about the same. The absolute values of these two sets of peaks appear to differ by somewhat less than 1 p.p.m. Were the monomer data recorded at 75°, these chemical shifts would be essentially the same. On the basis of the foregoing assumption for correlation of pyranoside carbon number of D-glucan chemical-shifts, the peak displacement for glycosidation appears to be less than that for methylation. Both the C-6 displacement (which can be unambiguously assigned) and the C-2 displacement are half the 10-p.p.m. chemical-shift ascribed to methylation.

Haverkamp *et al.*[17] discussed chemical shifts of per-*O*-methylated methyl glycosides of disaccharides, recorded in acetonitrile-d_3 at 30°, and compared the chemical-shift displacements for specific D-glucopyranoside carbon atoms when a methoxyl group is replaced with an *O*-α-D-glucosyl group; this is an alternative

method for obtaining the data abstracted in column 4 of Table IV. The data associated with α-(1→2)-, α-(1→4)-, and α-(1→6)-linkages are of interest [Haverkamp *et al.*[17] did not give α-(1→3)-linkage data]. The chemical shifts for α-D-glucosyl-bonded D-glucopyranosyl C-2 (Haverkamp *et al.*[17] for methyl per-*O*-methyl-α-kojibioside, 75.5 p.p.m.; this paper, 76.6 p.p.m.) and C-6 (Haverkamp *et al.*[17] for methyl per-*O*-methyl-α-isomaltoside, 67.0 p.p.m.; this paper, 66.6 p.p.m.) are in close agreement. However, the corresponding chemical-shifts for C-4 (Haverkamp *et al.*[17] for methyl per-*O*-methyl-α-maltoside, 73.3 p.p.m.; this paper, 79 p.p.m.) differ greatly. It is possible that this difference is due to extensive methylation, or the use of a nonaqueous solvent.

The 75–85-p.p.m. region appears to be diagnostic for determining the linkage type of α-D-linked D-glucans. Two D-glucans of each type of α-(1→2)-, α-(1→3)-, and α-(1→4)-linkage have been examined, with excellent correlation at 27° and pH 7. The α-(1→3)-linkage has a chemical shift at 81.6 p.p.m., the α-(1→4) at 79.5 p.p.m., and the α-(1→2) at 76.5 p.p.m. This is in close agreement with the published value[10] of 78.4 p.p.m. at 32° and pH 7. The percentages of non-α-(1→6)-linkages present appear to have little effect on the 75–85-p.p.m. region, and, for the α-(1→3)-linkage, there is apparently no difference between α-(1→3)-linked glycosyl residues and a 1,3,6-tri-*O*-substituted glycosyl residue. As previously noted, the chemical shift for cyclohexaamylose (82.1 p.p.m.) is unusually large for α-(1→4)-linked glycosyl residues.

Although ring-carbon positions have been ascribed to peaks A through F in Table IV for comparison purposes, this correlation should not necessarily be considered definitive; these positions are based on literature values for substituted D-glucans. It is possible that the only certain method for correlation of chemical shift to carbon position is to employ specific, deuterium substitution, as was done for a mannan by Gorin[18].

EXPERIMENTAL

Spectral conditions. — Proton-decoupled ^{13}C-n.m.r. spectra were obtained at natural abundance, with a total carbohydrate concentration of ~100 mg/2 ml of deuterium oxide. A Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 system was employed in the Fourier-transform, data-processing mode. The spectral width was 6 kHz; the acquisition time, 1.4 sec; and the pulse-width, 14 μsec. The number of transients was, in general, a function of the desired signal-to-noise ratio, and is described in Table III for each spectrum. The samples were measured in 12-mm diameter tubes spun at approximately 20 r.p.s. Temperatures were controlled to within ±1°. Sample temperatures were measured, before and after each experiment, to within ±0.5° with an immersion thermometer, and were held constant with a Varian temperature-controller. Chemical shifts are expressed in p.p.m. relative to external TMS, but were actually calculated by reference to the lock signal.

Materials. — The preparation and characterization of the dextrans[2] and dextran fractions[3] have been reported previously.

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REFERENCES

1 F. R. SEYMOUR, M. E. SLODKI, R. L. PLATTNER, AND A. JEANES, *Carbohydr. Res.*, 53 (1977), in press.
2 A. JEANES, W. C. HAYNES, C. A. WILHAM, J. C. RANKIN, B. E. MELVIN, M. J. AUSTIN, J. E. CLUSKEY, B. E. FISHER, H. M. TSUCHIYA, AND C. E. RIST, *J. Am. Chem. Soc.*, 76 (1954) 5041–5046.
3 C. A. WILHAM, B. H. ALEXANDER, AND A. JEANES, *Arch. Biochem. Biophys.*, 59 (1955) 61–75.
4 J. A. THOMA AND L. STEWART, in R. L. WHISTLER AND E. F. PASCHALL (Eds.), *Starch*, Vol. 1, Academic Press, New York, 1965, pp. 209–249.
5 T. USUI, N. YAMAOKA, K. MATSUDA, K. TUZIMURA, H. SUGIYAMA, AND S. SETO, *J. Chem. Soc. Perkin Trans. I*, (1973) 2425–2432.
6 Y. IWAKURA, K. UNO, F. TODA, S. ONOZUKA, K. HATTORI, AND M. L. BENDER, *J. Am. Chem. Soc.*, 97 (1975) 4432–4433.
7 H. J. JENNINGS AND I. C. P. SMITH, *J. Am. Chem. Soc.*, 95 (1973) 606–608.
8 I. C. P. SMITH, H. J. JENNINGS, AND R. DESLAURIERS, *Acc. Chem. Res.*, 8 (1975) 306–313.
9 D. E. DORMAN AND J. D. ROBERTS, *J. Am. Chem. Soc.*, 93 (1971) 4463–4472.
10 P. COLSON, H. J. JENNINGS, AND I. C. P. SMITH, *J. Am. Chem. Soc.*, 96 (1975) 8081–8087.
11 A. S. PERLIN, B. CASU, AND H. J. KOCH, *Can. J. Chem.*, 48 (1970) 2596–2606.
12 P. A. J. GORIN, *Can. J. Chem.*, 51 (1973) 2375–2383.
13 H.-J. SCHNEIDER, M. SCHOMMER, AND W. FREITAG, *J. Magn. Reson.*, 18 (1975) 393–397.
14 T. USUI, M. KOBAYASHI, N. YAMAOKA, K. MATSUDA, K. TUZIMURA, H. SUGIYAMA, AND S. SETO, *Tetrahedron Lett.*, (1973) 3397–3400.
15 M. TORII AND K. SAKABIBARA, *J. Chromatogr.*, 96 (1974) 255–257.
16 H.-J. SCHNEIDER AND W. FREITAG, *J. Am. Chem. Soc.*, 98 (1976) 478–481.
17 J. HAVERKAMP, M. J. A. DE BIE, AND J. F. G. VLIEGENTHART, *Carbohydr. Res.*, 37 (1974) 111–125.
18 P. A. J. GORIN, *Carbohydr. Res.*, 39 (1975) 3–10.